# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | 2004 | Journal Article-Journal of Applied Physiology |

4. TITLE AND SUBTITLE
Exertional Heat Injury and Gene Expression Changes: a DNA Microarray Analysis Study

5. FUNDING NUMBERS

6. AUTHOR(S)
L.A. Sonna, C.B> Wenger, S. Flinn, H.K. Sheldon, M.N. Sawka, C.M. Lilly

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Thermal & Mountain Medicine Division
U.S. Army Research Institute of Environmental Medicine
Kansas Street
Natick, MA 01760-5007

8. PERFORMING ORGANIZATION REPORT NUMBER
M03-35

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Same as #7 above.

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES



12a. DISTRIBUTION / AVAILABILITY STATEMENT

**DISTRIBUTION STATEMENT A**
Approved for Public Release
Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*
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| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| heat stroke; exercise; peripheral blood mononuclear cells; genomics | 11 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | |

*J Appl Physiol* 96: 1943–1953, 2004.
First published February 20, 2004; 10.1152/japplphysiol.00886.2003.

# Exertional heat injury and gene expression changes: a DNA microarray analysis study

**Larry A. Sonna,[1,2] C. Bruce Wenger,† Scott Flinn,[3] Holly K. Sheldon,[2] Michael N. Sawka,[1] and Craig M. Lilly[2]**

[1]*Thermal and Mountain Medicine Division, United States Army Research Institute of Environmental Medicine, Natick 01760;* [2]*Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital/Harvard Medical School, Boston, Massachusetts 02115; and* [3]*Directorate for Primary Care, Naval Medical Center San Diego, San Diego, California 92134*

Submitted 19 August 2003; accepted in final form 9 January 2004

**Sonna, Larry A., C. Bruce Wenger, Scott Flinn, Holly K. Sheldon, Michael N. Sawka, and Craig M. Lilly.** Exertional heat injury and gene expression changes: a DNA microarray analysis study. *J Appl Physiol* 96: 1943–1953, 2004. First published February 20, 2004; 10.1152/japplphysiol.00886.2003.—This study examined gene expression changes associated with exertional heat injury (EHI) in vivo and compared these changes to in vitro heat shock responses previously reported by our laboratory. Peripheral blood mononuclear cell (PBMC) RNA was obtained from four male Marine recruits (ages 17–19 yr) who presented with symptoms consistent with EHI, core temperatures ranging from 39.3 to 42.5°C, and elevations in serum enzymes such as creatine kinase. Controls were age- and gender-matched Marines from whom samples were obtained before and several days after an intense field-training exercise in the heat ("The Crucible"). Expression analysis was performed on Affymetrix arrays (containing ~12,600 sequences) from pooled samples obtained at three times for EHI group (at presentation, 2–3 h after cooling, and 1–2 days later) and compared with control values (average signals from two chips representing pre- and post-Crucible samples). After post hoc filtering, the analysis identified 361 transcripts that had twofold or greater increases in expression at one or more of the time points assayed and 331 transcripts that had twofold or greater decreases in expression. The affected transcripts included sequences previously shown to be heat-shock responsive in PBMCs in vitro (including both heat shock proteins and non-heat shock proteins), a number of sequences whose changes in expression had not previously been noted as a result of in vitro heat shock in PBMCs (including several interferon-induced sequences), and several nonspecific stress response genes (including ubiquitin C and dual-specificity phosphatase-1). We conclude that EHI produces a broad stress response that is detectable in PBMCs and that heat stress per se can only account for some of the observed changes in transcript expression. The molecular evidence from these patients is thus consistent with the hypothesis that EHI can result from cumulative effects of multiple adverse interacting stimuli.

heat stroke; exercise; peripheral blood mononuclear cells; genomics

EXERTIONAL HEAT ILLNESS encompasses a spectrum of clinical conditions, of which the most severe are exertional heat injury (EHI) and exertional heatstroke (EHS). EHI is defined as severe illness associated with high body temperatures in the setting of physical exertion, typically involving exhaustion or collapse, which is accompanied by evidence of tissue and organ damage (11). EHS is a more severe form of heat illness defined clinically by the presence of significant concurrent central nervous system dysfunction (11). EHI and EHS account for many medical emergencies each year in athlete, worker, and military populations (25) and have recently received considerable attention because of a number of high-profile deaths among athletes.

The pathophysiological mechanisms that produce EHI and EHS are under active investigation (2). Experimental approaches have included investigations of animal mortality (10, 14), tissue damage (13, 17), and cellular factors [such as heat shock protein (HSP) expression] that affect tissue susceptibility to thermal injury (9, 16, 20). Animal models and observations in humans have identified mechanisms such as heat-induced translocation of lipopolysaccharide from the bowel lumen into the circulation (2, 12, 17, 22), reticuloendothelial system deactivation (4), and heat-induced changes in pro- and anti-inflammatory cytokines (2) as contributing to the pathogenesis of EHI and EHS. Additionally, human studies have examined the possibility that an inflammatory response induced by previous muscle injury leads to an accentuated hyperthermic response during exercise-induced heat loads (19). The picture that emerges from these mechanistic studies is that EHI and EHS share a common pathophysiological basis (3, 11) and represent systemic manifestations of derangements that occur at the level of cells and tissues, in which the immune system is highly involved (2). Identification of cellular pathways involved in EHI and EHS is a logical and important extension of this body of work. Until recently, this has been difficult because of limitations in our ability to simultaneously assay large numbers of mediator molecules in the context of thermal stress.

At the cellular level, it is generally accepted that thermal stress leads to increases in expression of HSPs and that the expression of these proteins correlates closely with the acquisition of thermotolerance (16, 18, 21). However, it is also increasingly apparent that the cellular response to heat shock involves more than HSPs (16, 28). For example, a recent DNA microarray study of rats exposed to whole body hyperthermia found substantial changes in liver expression of both HSPs and genes belonging to other functional classes (32). Similarly, isolated human peripheral blood mononuclear cells (PBMCs) subjected to in vitro heat shock demonstrated extensive changes in expression of both HSPs and genes not traditionally considered as HSPs (29).

†Deceased 22 November 2002.

Address for reprint requests and other correspondence: L. A. Sonna, Thermal and Mountain Medicine Div., USARIEM, Natick, MA 01760 (E-mail: larry.sonna@na.amedd.army.mil).

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Knowledge of the molecular processes involved in EHI and EHS in humans has lagged behind our understanding of systemic processes, in part because it is difficult to obtain samples of human tissues thought to be of high potential interest, such as intestine, liver, skeletal muscle, and heart. Because leukocytes are easily obtained from human volunteers and show changes in HSP expression after heat shock in vitro (6, 7, 23, 24, 29) and after exercise in vivo (6–8, 23, 24), and because blood is exposed to all body compartments and thus all body temperatures (which are typically substantially elevated during EHI and EHS), PBMCs likely represent an informative cell type to identify molecular changes in EHI and EHS patients. Accordingly, we thought it likely that individuals suffering from EHI would demonstrate substantial changes in PBMC gene expression and that at least some of these changes would be similar to those found in human in vitro models of heat shock. This study, therefore, examined in vivo changes in PBMC gene expression associated with EHI and compared them to in vitro heat shock responses in PBMCs that were previously reported by our laboratory (29). We hypothesized that gene expression would be similar to heat shock but that many other genes associated with known systemic processes, particularly those related to inflammatory responses, would be expressed with EHI.

## MATERIALS AND METHODS

*Subjects and controls.* The four EHI cases were male Marine recruits undergoing basic training at Parris Island, who presented to the field corpsman (medic) with evidence of heat illness and were evacuated to the Branch Medical Clinic Parris Island. The criteria for EHI included hyperthermia from physical exercise, with signs and symptoms such as collapse during exertion in the heat, malaise, disorientation, or ataxia, accompanied by laboratory evidence of organ dysfunction [such as elevated serum creatine kinase (CK), transaminases, or lactate dehydrogenase], a level of consciousness that returned to normal after cooling, and the absence of seizure activity. All subjects had active cooling started in the field (icing). On arrival to the clinic, they underwent further active cooling with ice sheets, typically followed by showering. In addition, all subjects received intravenous hydration. Blood samples were drawn on presentation, after cooling [mean ± SD, 3:02 ± 0:24 (h:min) after presentation], and again on a follow-up visit 1–2 days later. Several blood samples were drawn on *case 4* in the days after the initial scheduled follow-up. All cases were presented between July and September 2000. All four subjects had been in basic training at Parris Island long enough (range, 9–20 days from start of training to injury) to have become heat acclimatized.

Control subjects were three Marine recruits matched for age, gender, and ethnic group, who volunteered to donate blood samples in August 2000, several days before and several days after an intense field-training exercise ("The Crucible," the capstone event of US Marine Basic Training, which represents 54 h of vigorous field training with average energy expenditures of >6,000 kcal/day for men). Because this event was preceded by weeks of basic combat training in the summer heat, it is assumed that the controls were also heat acclimatized.

All Marine Corps recruits are medically screened before enlistment for an extensive battery of disqualifying conditions, including a variety of chronic liver diseases, infectious diseases such as chronic hepatitis, autoimmune conditions such as systemic lupus erythematosus that can produce renal and hepatic dysfunction, autoimmune conditions capable of producing rhabdomyolysis, alcohol dependence, and renal disease. Also disqualifying for enlistment are a known predisposition to heat illness, a prior history of malignant hyperthermia, recurrent episodes of heat injury requiring medical attention, and evidence of residual injury from a prior heat illness.

*Blood collection.* Blood was collected from all individuals in cell-preparation tubes (Fisher Scientific). Mononuclear cells were separated per the manufacturer's instructions, suspended in ~1 ml of RNALater (Ambion), and frozen at −80°C until RNA processing occurred.

*RNA extraction.* Samples were thawed, and the cells were pelleted by centrifugation. RNA was extracted by using the RNeasy Mini Kits (Qiagen). RNA yield was estimated by absorbance spectrophotometry, and the samples were stored at −80°C.

*DNA microarray analysis.* Due to limiting sample volumes and RNA yields, equal amounts of RNA from the samples obtained from the four cases were pooled. Similarly, equal amounts of RNA from the samples obtained from the three control subjects were pooled. DNA microarray analysis was performed by using Affymetrix U95Av2 gene chips as described previously (27, 29). Analyses using Affymetrix arrays (containing ~12,600 sequences) were performed from pooled samples obtained at each of the three time points for EHI group (1 chip each for samples obtained at presentation, after cooling, and at follow-up) and compared with control values (1 chip each for samples obtained before and after the Crucible) as described in *Statistical analysis*.

*Expression measurement by RT-PCR.* RT-PCR on the pooled samples was performed using standard techniques, as described previously (27, 29). The primers used for these reactions are listed (see Table 4).

*Statistical analysis.* The output signal from the Affymetrix U95Av2 array was preprocessed using MAS 5.0 software (Affymetrix). For each sequence on the array, the mean and standard deviation of the software-reported signals were calculated from the two pooled control chips. From these values, 98.33% population intervals were computed by adding or subtracting 2.39 standard deviations from the mean. Corresponding sequences from the heat-injury cases were considered to have a statistically significant deviation from the controls if their signals fell outside of these population intervals. Population intervals of 98.33% were chosen rather than 95% intervals to correct for the three multiple comparisons being performed; i.e., a $P$ value of $0.05/3 = 0.0167$ was considered statistically significant. Other statistical analyses were performed as noted throughout the manuscript using SigmaStat 2.03 for Windows and taking a $P$ value of $\leq 0.05$ as statistically significant.

Where noted in this manuscript, post hoc filtering of significantly affected sequences was performed by two criteria. First, sequences had to show a twofold or greater change in expression. Second, sequences had to be detected by the chip-reading software as "present" or "marginal" in both control chips (for downregulated sequences) or in the chip(s) corresponding to the time point(s) of interest (for upregulated sequences). This post hoc filtering was performed to maximize the comparability of the results of this experiment with the analysis performed in our laboratory's previous in vitro experiment (29) involving five sets of normal human PBMCs subjected to heat shock in serum-free media. In this previous study, preprocessing was performed using MAS 4.0 software, and the post hoc filters used were a geometric mean change in expression of twofold or greater and a present or marginal call in five of five experiments in the controls (for downregulated sequences) or in the heat-shocked cells (for upregulated sequences).

## RESULTS

*EHI and control subjects.* Table 1 provides the demographic characteristics of the EHI and control subjects. Tables 2 and 3 provide the clinical and laboratory characteristics of the EHI patients. Values outside the range of normal for the clinical laboratory are highlighted in bold font in Table 3.

Table 1. *Demographic characteristics of cases and controls*

| | Ethnic Origin | Age, yr | Height, m | Weight, kg | BMI, kg/m² | Time Between Presentation and Follow-up, days |
|---|---|---|---|---|---|---|
| Cases | | | | | | |
| *1* | Caucasian | 17 | 1.75 | 68.5 | 22.3 | 2 |
| *2* | African-American | 17 | 1.70 | 81.6 | 28.2 | 1 |
| *3* | African-American | 18 | 1.83 | 100.2 | 30.0 | 2 |
| *4* | Caucasian | 19 | 1.83 | 89.4 | 26.7 | 1 |
| Mean ± SE | | 18 ± 0.5 | 1.78 ± 0.03 | 84.9 ± 6.7 | 26.8 ± 1.6 | 1.5 ± 0.3 |
| Controls | | | | | | |
| A | African-American | 19 | 1.71 | 60.8 | 20.7 | 10 |
| B | Caucasian | 18 | 1.82 | 80.7 | 24.5 | 14 |
| C | Caucasian | 18 | 1.78 | 79.4 | 25.1 | 14 |
| Mean ± SE | | 18 ± 0.3 | 1.77 ± 0.03 | 73.6 ± 6.4 | 23.4 ± 1.4 | 13 ± 1 |
| *P* value | 1.0* | 0.40 | 0.88 | 0.29 | 0.20 | <0.001 |

BMI, body mass index. *By Fisher's exact test. *P* values were derived using *t*-tests unless otherwise indicated.

All four EHI cases had at least one recorded body temperature in excess of 102.2°F (39°C) with their highest core temperatures ranging from 102.8°F (39.3°C) to 108.5°F (42.5°C) (as recorded either in the field or at the branch medical clinic). All subjects received initial cooling in the field with ice sheets and up to 14 min of additional treatment with ice sheets in the cool room of the acute care treatment area of the Branch Medical Clinic (Table 2).

Each of four EHI cases had prodromal symptoms in the days leading up to the acute event. The precipitating event involved running in three of the four cases and participation in an obstacle course in the fourth. No subject had neurological impairment more severe than mild confusion. All subjects had at least one blood sample showing elevation of unfractionated CK. With the exception of the fourth case, none of the cases had evidence of urinary heme pigment on the day of injury as judged by urine dipstick test. *Case 4* had both a positive urinary dipstick test for heme pigment and a minor degree of microscopic hematuria on presentation (with 10–25 erythrocytes per high power field on urinalysis). *Case 1* had evidence of mononucleosis (based on elevated serum Epstein Barr virus viral capsid IgM levels), *case 3* had pharyngeal/tonsillar erythema and exudates on exam, and the chest radiograph from *case 4* was read by the treating practitioners as having an infiltrate in the left lower lobe that was felt to be suspicious for pneumonia. All cases displayed elevations of serum CK above normal, although only *case 4* displayed an elevation of CK above 3,000, suspicious for clinically significant rhabdomyolysis. All four cases received intravenous hydration and showed a resulting decrease in serum creatinine. There were varying degrees of leukocytosis, with a relative shift in the distribution of cell types toward predominance of granulocytes (>80%) in the blood samples taken after cooling (Table 3). In summary, each of our case subjects met the definition of EHI, although a broad range of disease severity was represented.

*Number of sequences affected by EHI.* Of the ~12,600 sequences present on the U95Av2 chip, 3,605 and 4,478 sequences were expressed as "present" or "marginal" in the pooled controls (drawn before and after the field-training exercise, respectively). In the cases, 4,040 sequences were similarly expressed at presentation to the acute care treatment room, 4,056 were expressed after cooling, and 3,634 were expressed at the time of follow-up. These numbers are consistent with the number of sequences consistently expressed in previous in vitro experiments with human PBMCs (~3,700) that used an earlier version of the U95 array (29).

Table 2. *Clinical characteristics of subjects experiencing exertional heat illness*

| Case | Prodromal Symptoms | Activity Precipitating EHI | Level of Consciousness (Initial Field Examination) | Maximum Recorded Temperature* °F | Maximum Recorded Temperature* °C | BMC Time on Ice Sheets, min† | Temperature at Time of Removal From Ice* °F | Temperature at Time of Removal From Ice* °C | Hydration in BMC, liters | Other Pertinent Findings |
|---|---|---|---|---|---|---|---|---|---|---|
| *1* | URI × 3 days, nausea | 2-mile run | Normal | 103.2 | 39.6 | 9 | 101.6 | 38.7 | IV: 2–3 | Positive serum Epstein-Barr virus viral capsid IgM |
| *2* | URI × 2 days | Obstacle course | Slowed/confused | 102.8‡ | 39.3 | 10 | 102.3 | 39.1 | IV: 2–3 PO: 1 | |
| *3* | URI × 4–5 days | Training run | Normal | 103.8 | 39.9 | 6 | 101.7 | 38.7 | IV: 2 PO: 0.75 | Pharyngeal/tonsillar erythema and exudates |
| *4* | Fever × 2 days; fatigue for several days | 3-mile run of the physical fitness test | Slowed/confused | 108.5 | 42.5 | 14 | 102.5 | 39.2 | IV: 2 | Left-sided wheezing; left lower lobe pulmonary infiltrate suspicious for pneumonia on chest X-ray |
| Mean±SD | | | | 104.6 ±2.6 | 40.3 ±1.5 | 10±3 | 102.0 ±0.4 | 38.9 ±0.2 | | |

BMC, Branch Medical Clinic; URI, upper respiratory illness; IV, intravenous; PO, oral; IgM, immunoglobulin M. *Oral or rectal; medical records did not always specify which. Maximum temperatures were recorded in the field except as noted. †In all cases, subjects were wetted down and treated with ice sheets in the field. ‡Recorded on arrival in the BMC; no field temperature was recorded.

Table 3. *Clinical laboratory characteristics of subjects experiencing exertional heat illness*

| Case | Maximum Serum Creatine Kinase | | Serum Creatinine, mg/dl* | | | Maximum Serum AST | | Maximum Serum ALT | | Maximum Serum LDH | | Serum Glucose, mg/dl | | | WBC Counts and Differentials | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | | At presentation | | | After cooling | | | At follow-up | | |
| | Value, IU/l | Occurred on | On presentation | After cooling | At follow-up | Value, IU/l | Occurred on | Value, IU/l | Occurred on | Value, IU/l | Occurred on | On presentation | After cooling | At follow-up | WBC count, $10^3$/mm$^3$ | Granulocytes, % | PBMCs, % | WBC count, $10^3$/mm$^3$ | Granulocytes, % | PBMCs, % | WBC count, $10^3$/mm$^3$ | Granulocytes, % | PBMCs, % |
| 1 | 1,844 | Injury day | 1.4 | 1.1 | 1.3 | 63 | Injury day | 26 | Injury day | 311 | Injury day | 114 | 93 | 110 | 11.2 | 38.7 | 61.3 | 8.0 | 88.7 | 11.3 | 6.8 | 53.1 | 46.9 |
| 2 | 514 | Follow-up | 1.4 | 1.1 | 1.0 | 40 | Follow-up | 29 | Follow-up | 269 | Injury day | 136 | 83 | 90 | 17.7 | 72.7 | 26.6 | 16.7 | 82.8 | 16.9 | 14.8 | 84.0 | 16.0 |
| 3 | 412 | Injury day | 1.2 | 0.9 | 0.9 | 44 | Injury day | 23 | Injury day | 237 | Injury day | 89 | 85 | 94 | 5.3 | 62.5 | 37.5 | 6.7 | 80.9 | 19.1 | 5.5 | 67.8 | 32.2 |
| 4 | 10,639 | 2 days after follow-up | 2.0 | 1.4 | 1.0 | 628 | Follow-up | 642 | Day after follow-up | 1,010 | Follow-up | 163 | 95 | 102 | 18.1 | 76.4 | 23.6 | N/A | N/A | N/A | 8.2 | 82.7 | 17.3 |

AST, asparate aminotransferase; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; WBC, white blood cell; PBMCs, peripheral blood mononuclear cells; N/A, not available. Ranges of normal for this reference laboratory are: creatinine kinase, 22–269 IU/l; creatinine, 0.5–1.5 mg/dl; serum AST, 10–42 IU/l; serum ALT, 10–60 IU/l; serum LDH, 100–242 IU/l; serum glucose, 70–110 mg/dl; WBC count, 4.0–11.0 × $10^3$/mm$^3$; % granulocytes, 42.2–75.2%. Values outside the range of normal for the clinical laboratory used are in bold. *Changes over time were statistically significant by one-way, repeated-measures ANOVA ($P = 0.038$).

Although the absolute number of sequences expressed during EHI did not differ significantly from those expressed at baseline, the number of sequences that showed significantly different expression between cases and controls was large. As shown in Table 5, ~1,800 sequences showed a statistically significant increase in expression at each of the time points examined in the subjects with EHI, and ~2,000 sequences showed a significant decrease in expression. However, most of these changes in expression were relatively small, with only ~800 sequences showing increases of twofold or greater at each of the time points and a similar number showing decreases (Table 5). Even fewer sequences met our presence/absence post hoc filter criteria; in total, we estimate that 361 sequences were upregulated at one or more time points and that 331 sequences were downregulated.

The sequences that were significantly affected by EHI changed as a function of time. As illustrated by the Venn diagrams in Fig. 1 (center of each diagram), only a minority of sequences were significantly upregulated (or downregulated) at all three time points. A substantial number sequences showed a time-dependent response; for example, HSPs were typically most highly upregulated on the day of injury and had diminished or returned to control levels by the time of follow-up (Table 6).

We classified all sequences that were significantly affected and met our post hoc filter criteria into broad functional classes. For purposes of classification and illustration, each sequence was assigned to only one primary functional class. Of the 361 upregulated sequences, about three-fourths fell into one of the following classes: immune function, 85 sequences (24%); cell growth, proliferation, differentiation, and apoptosis, 37 sequences (10%); metabolism and redox control (including heme oxygenase-1), 33 sequences (9%); transcription, 29 sequences (8%); unknown, 29 sequences (8%); protein degradation (including ubiquitins, proteases, and antiproteases), 23 sequences (6%); HSPs, chaperonins, and cochaperonins, 21 sequences (6%); and signal transduction, 22 sequences (6%). Of the 331 downregulated sequences, about two-thirds fell into one of the following classes: unknown, 58 sequences (18%); immune function, 42 sequences (13%); cell growth, proliferation, differentiation, and apoptosis, 44 sequences (13%); transcription, 41 sequences (12%); signal transduction, 32 sequences (10%); and metabolism and redox control, 18 sequences (5%).

*Heat shock response.* EHI produced a strong, time-dependent heat shock response in PBMCs. As shown in Table 6, a significant change in expression was found in at least one representative member of most major families of known human HSP. As might be expected from a heat-induced process, the number of significantly upregulated HSP sequences was greatest at presentation and diminished over time. At the time of follow-up, only eight HSP sequences were still significantly upregulated, and, of these, only two showed a change in expression that was twofold or more greater than controls. The finding of a widespread increase in HSP expression is highly congruent with our laboratory's previous microarray study of the effect of in vitro heat shock on gene expression in PBMCs (29), and as noted in Table 6, almost all of the HSP sequences upregulated by EHI in vivo had previously been found to be upregulated in our in vitro heat shock experiments.

Table 4. *PCR Primers used*

| Gene | GenBank No. | PCR Product Size, bp | Forward Primer | Reverse Primer |
|---|---|---|---|---|
| PSR | AI950382 | 345 | 5′-GCATTGGTAGCAGAGGGAAA-3′ | 5′-GCTCCAGTTCGTCAGACTCC-3′ |
| IFI-60 | AF026939 | 427 | 5′-AGGAAGGGTGGACACAACTG-3′ | 5′-TAGCCATTGTTTGGTGTGGA-3′ |
| IFI-27 | X67325 | 305 | 5′-GCCTCTGCTCTCACCTCATC-3′ | 5′-GAGAGTCCAGTTGCTCCCAG-3′ |
| Unknown transcript | AB000115 | 321 | 5′-TCAGATTTGGAACTGGACCC-3′ | 5′-TCAGGTTTTGGCCTTTGAAC-3′ |
| HSP70B′ | X51757 | 579 | 5′-TGCCCGCCTATTTCAATG-3′ | 5′-CCCCCACCAGGACGAC-3′ |
| Cyclophilin A | BC005982 | 406 | 5′-AGGTCCCAAAGACAGCAGAA-3′ | 5′-TGTCCACAGTCAGCAATGGT-3′ |

PSR, phosphatidylserine receptor; IFI, interferon inducible; HSP, heat shock protein.

*Control sequences.* Although EHI produced extensive changes in gene expression, these changes in expression were not universal. Table 7 shows the effect of EHI on a number of control sequences, including β-actin, GAPDH, cyclophilin A, and the five ribosomal protein sequences most highly expressed in the control subjects. Although some of these sequences showed changes in expression that were significantly different from the controls, none showed an absolute change of twofold or greater, the cutoff used in our post hoc filtering process.

*Effect of EHI on non-HSPs.* The non-HSPs most strongly (≥5.0-fold after rounding, at one or more time points) affected by EHI are listed in Tables 8 and 9. Importantly, of the 36 upregulated sequences listed in Table 8, at least one-fourth are known to be interferon inducible. Interestingly, in comparing these results to previous in vitro data (29), only two of these non-HSPs had previously been found to be significantly upregulated after an in vitro heat shock delivered to normal PBMCs (43°C × 20 min followed by 2 h 40 min of recovery at 37°C): the phosphatidylserine receptor (upregulated 3.1-fold in vitro) and pyruvate carboxylase (upregulated 1.8-fold in vitro but did not meet our post hoc filter criteria in the previous study). Furthermore, seven of these upregulated genes were actually downregulated in our previous in vitro experiment: granzyme B (down 0.49-fold in vitro), adrenomedullin (down 0.47-fold in vitro), complement component 3a receptor 1 (down 0.40-fold in vitro), myxovirus resistance A (down 0.23-fold in vitro), endothelial cell growth factor 1 (down 0.38-fold in vitro), interferon inducible protein 60 (down 0.12-fold in vitro), and interferon-induced protein with tetratricopeptide 2 (down 0.22-fold in vitro but did not meet the previous study's post hoc filter criteria). Among the differences between the two experiments, it is noteworthy that the in vitro experiment was performed in serum-free media.

Table 5. *Number of sequences affected by exertional heat illness*

| Time Point | Direction of Change | Number of Sequences Showing: Statistically significant difference in expression* | ...and a signal difference of ≥2-fold* | ...and also met our post hoc presence/absence filter criteria |
|---|---|---|---|---|
| At presentation | Any | 3615 | | |
| | Up | 1786 | 767 | 144 |
| | Down | 1829 | 717 | 115 |
| After cooling | Any | 3900 | | |
| | Up | 1742 | 760 | 237 |
| | Down | 2158 | 966 | 195 |
| At follow-up | Any | 3923 | | |
| | Up | 1895 | 856 | 179 |
| | Down | 2028 | 917 | 176 |
| At any time point | Any† | 5558 | | |
| | Up | 3147 | 1555 | 361 |
| | Down | 3179 | 1675 | 331 |

*Relative to matched controls. †Total does not equal the sum of "up" plus "down," because some sequences showed a biphasic response (e.g., up at 1 or more time points, down at others).

A sequence-encoding interferon-γ (GenBank no. X13274) was significantly upregulated by EHI, with changes of 3.7-fold at presentation, 3.0-fold after cooling, and 7.6-fold at follow-up. However, this sequence showed a low level of expression and did not meet our post hoc presence/absence filter criteria. In our laboratory's previous in vitro experiment (29), this sequence was upregulated 4.3-fold, but the variance from experiment to experiment was high enough to render the change not statistically significant (increased in 4 experiments, decreased in 1 experiment; 95% confidence interval, 0.66 to 28). Thus, both in vitro and in vivo, our microarray evidence is equivocal but favors the possibility that heat stress leads to increased expression of interferon-γ mRNA.

Two interferon-α sequences on the Affymetrix array (GenBank V00541 and X02956, both encoding interferon-$\alpha_5$) showed statistically significant increases of twofold or greater at presentation (2.4-fold and 2.1-fold, respectively) but not after cooling or at recovery. However, neither of these sequences met our post hoc criteria for inclusion, and neither had been upregulated in our laboratory's previous in vitro study (29).

Among the 35 most strongly downregulated sequences, only 5 were previously found to be downregulated as a result of in vitro PBMC heat shock (Table 9). It is noteworthy that the time point at which maximum downregulation occurred was most commonly at follow-up (1–2 days after initial presentation), which is substantially later than the time point examined in the in vitro experiment (2 h 40 min after heat shock).

*Confirmatory RT-PCR.* RT-PCR was performed on a select number of genes identified by the microarray as being strongly upregulated by heat shock (Fig. 2). The source material for this PCR came from control *subject B* (*lanes 1* and *2*) and *case 2* (*lanes 3–5*). As illustrated, the PCR confirmed the presence of an increased expression of HSP70B′, phosphatidylserine receptor, interferon-induced proteins 27 and 60, and AB000115 (an open reading frame on chromosome 1) in the EHI case. By contrast, there was no clear effect on cyclophilin A, as predicted by the microarray results.

*Comparison to previous in vitro heat shock in PBMCs.* Previous work in our laboratory (27) has suggested that human cells display a relatively small nonspecific response to environmental stress in addition to stress- and cell type-specific

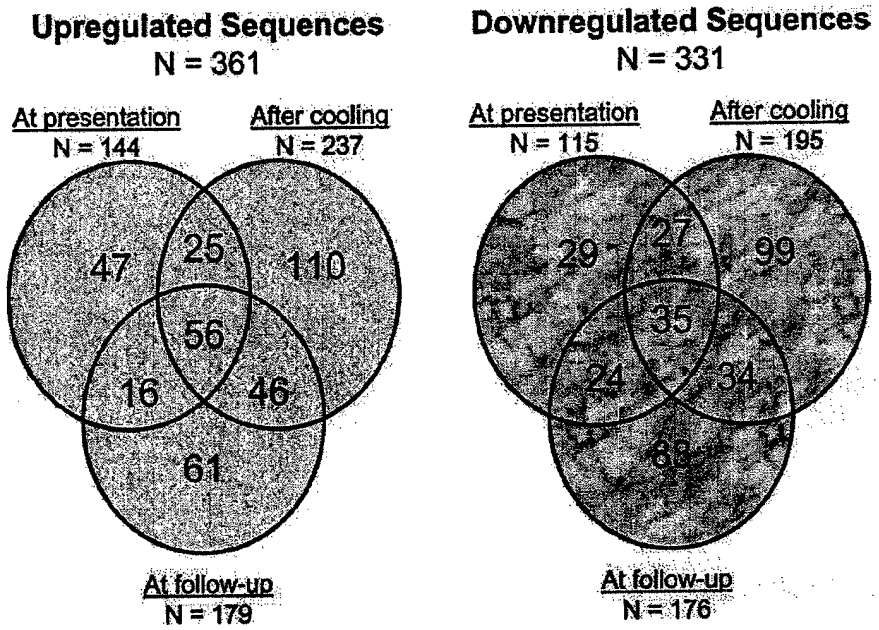


Fig. 1. Differentially expressed sequences grouped by time after exposure for significantly unregulated (*left*) and downregulated (*right*) sequences.

responses. From the data in Tables 6, 8, and 9, it became apparent that the PBMCs of the subjects with EHI had evidence of not only a conventional heat shock response (defined as increased expression of HSPs and other genes previously found to be upregulated by heat shock in vitro) but also of a response that differed from the previously observed in vitro effects of heat shock on PBMCs. To better estimate the extent to which our in vivo responses could be accounted for by thermal stress alone, we compared the list of 361 upregulated and 331 downregulated genes to the list of all genes significantly affected by heat shock in our in vitro PBMC experiment (29). Similarly, to estimate the magnitude of the stress nonspecific response (previously estimated at ~10–15% of all sequences affected), we compared the changes in expression noted here to those found in a database of cells exposed to a nonthermal stress (HepG2 cells exposed to hypoxia in vitro for 24 h) (27). To reduce the biasing effect of post hoc filtering, we included all sequences from the in vitro experiments that showed a statistically significant change in expression, independent of the magnitude of change or the presence/absence calls.

Of the 144 sequences upregulated at presentation, 35 (24%) had previously been found to be significantly upregulated by in vitro heat shock. By contrast, only 13 (9%) were similarly affected by hypoxic exposure in HepG2 cells ($P < 0.001$ by $\chi^2$ analysis). Of the 237 sequences upregulated after cooling, 41 (17%) were similarly affected by in vitro heat shock, which was comparable to the number of sequences affected by hypoxia [34 (14%), $P = 0.45$]. When we compared the 179 sequences that were upregulated at recovery, we found that only 17 (9%) were similarly affected by in vitro heat shock and 15 (8%) by hypoxia ($P = 0.85$). Although these numerical findings would suggest an overlap between the three studies of ~10–15%, when the actual sequences affected were compared, only eight sequences representing seven unique genes were significantly upregulated in all three studies at one or more time points. These included the well-established stress genes ubiquitin C, dual-specificity phosphatase-1, and colligin 2 (HSP47), as well as the phosphatidylserine receptor, calcium-binding protein S100A2, pyruvate carboxylase, and the collagen synthesis enzyme lysine hydroxylase.

Among the downregulated sequences, 11–18% have previously been found to be significantly downregulated by in vitro heat shock in PBMCs or by hypoxic exposure in HepG2 cells. Neither of the two in vitro experiments showed a significantly greater degree of overlap than the other with the effects of EHI.

## DISCUSSION

Our most important finding is that the PBMC gene expression response to EHI is far more extensive than previously recognized. This response appears to include a time-dependent series of changes that has at least three components: *1*) a heat shock response that involves elements such as HSPs, as described in PBMCs and other cell lines in vitro; *2*) a response that includes a number of genes not previously found to be upregulated by heat shock in our in vitro experiments in PBMCs and that includes a substantial number of interferon-inducible genes; and *3*) a small nonspecific stress response that is shared by other cell lines and stressors.

Although limited by several technical considerations (discussed below), the findings reported here are biologically plausible within the context of what is known about thermal stress in vitro and in vivo. For example, a time-dependent heat shock response was observed, a phenomenon that has been noted in vivo in contexts such as intense exercise (7, 8). It is also noteworthy that a sequence corresponding to c-myc was among the most highly downregulated sequences at presentation, because in vitro experiments have found that decreased expression of this gene is essential to the process of cellular recovery from severe thermal stress (31). Additionally, the congruence between the findings of the present study and our

Table 6. *HSPs affected by exertional heat illness*

| Family | Common Name(s) | GenBank or TIGR No. | Fold-Change in Expression (Subjects/Controls) | | | Effect of In Vitro Heat Shock |
|---|---|---|---|---|---|---|
| | | | At presentation | After cooling | At follow-up | |
| HSP10 | HSPE1; heat shock 10-kDa protein 1; chaperonin 10; HSP10 | AI912041 | 2.3 | 3.8 | 0.40 | Up |
| HSP27 | HSPB1; Heat shock 27-kDa protein 1; HSP28; HSP27-1 | Z23090 | 9.6 | 32 | 1.1 (NS) | Up |
| HSP32 | Heme oxygenase-1; HSP32 | Z82244 | 3.8 | 3.3 | 1.4 | Up |
| HSP40 | DNAJA1; DnaJ (HSP40) homolog, subfamily A, member 1; HSPF4 | L08069 | 3.5 | 1.4 (NS) | 1.2 (NS) | Up |
| | DNAJA1; DnaJ (HSP40) homolog, subfamily A, member 1; HSPF4 | L08069 | 2.1 | 1.3 (NS) | 0.93 (NS) | Up |
| | DNAJB1; DnaJ (HSP40) homolog, subfamily B, member 1; Hsp40 | D85429 | 8.4 | 1.5 (NS) | 0.71 (NS) | Up |
| | DNAJB6; DnaJ (HSP40) homolog, subfamily B, member 6; MRJ | AI540318 | 2.5 | 1.9 | 1.7 | Up* |
| HSP47 | HSP47; colligin 2; SERPINH2 | D83174 | 6.6 | 7.4 | 0.66 (NS) | Up |
| | HSP47; colligin 2; SERPINH2 | D83174 | 1.5 | 1.6 | 0.40 | Up |
| HSP56 | FKBP4; FK506 binding protein 4, 59-kDa; immunophilin; FKBP52; HSP56 | M88279 | 5.2 | 6.7 | 1.6 (NS) | Up |
| HSP60 | HSPD1; Heat shock 60-kDa protein 1 (chaperonin); HSP60-1; mitochondrial matrix protein P1 | M22382 | 3.3 | 5.4 | 0.85 (NS) | Up |
| HSP70 | HSPA1A and HSPA1B; Heat shock 70-kDa protein 1A; HSP70-1; heat shock 70-kDa protein 1B; HSP70-2 | W28645 | 2.9 | 2.0 | 1.3 (NS) | Up |
| | HSPA1A; heat shock 70-kDa protein 1A; HSP70-1 | M11717 | 31 | 22 | 1.2 | Up |
| | HSPA1B; Heat shock 70-kDa protein 1B; HSP70-2 | M59830 | 32 | 19 | 1.1 (NS) | Up |
| | HSPA6; heat shock 70-kDa protein 6; HSP70-B′ | X51757 | 25 | 3.6 | 3.8 | Up |
| | HSPA6; heat shock 70-kDa protein 6; HSP70-B′ | X51757 | 20 | 3.8 | 2.3 | Up |
| | HSPA8; heat shock 70-kDa protein 8; HSC70 | HT2995 | 2.9 | 1.6 (NS) | 0.85 (NS) | Up |
| | HSPA8; heat shock 70-kDa protein 8; HSC70 | HT2995 | 2.0 | 1.1 (NS) | 0.80 | Up |
| | HSPA9B; heat shock 70-kDa protein 9B; mortalin 2 | L15189 | 0.81 | 0.86 (NS) | 0.47 | Up* |
| HSP75 | TRAP1; heat shock protein 75; HSP75; tumor necrosis factor type 1 receptor associated protein | U12595 | 0.54 | 0.39 | 0.89 (NS) | NS |
| HSP90 | HSPCA; heat shock 90-kDa protein 1, alpha; HSP90-1-alpha | X15183 | 5.8 | 6.8 | 1.1 (NS) | Up |
| | HSPCB; heat shock 90-kDa protein 1, beta; HSP 90-1-beta; HSP90B | J04988 | 3.1 | 3.3 | 0.76 | Up |
| | HSPCB; heat shock 90-kDa protein 1, beta; HSP 90-1-beta; HSP90B | M16660 | 2.6 | 2.8 | 0.68 (NS) | Up |
| Other | FKBP5; FK506 binding protein 5; HSP90-binding immunophilin | U42031 | 0.68 (NS) | 2.1 | 1.2 (NS) | Up* |
| | STIP-1; HSP70/HSP90 organizing protein; stress-induced phosphoprotein 1 | M86752 | 2.2 | 2.2 | 1.2 (NS) | Up |
| Ubiquitins | UBB; ubiquitin B | U49869 | 2.1 | 1.4 (NS) | 1.8 | Up |
| | UBC; ubiquitin C | M26880 | 2.8 | 2.2 | 1.7 | Up* |
| | UBC; ubiquitin C | AB009010 | 2.3 | 1.8 | 1.6 | Up* |
| Upregulated | | | 25 | 20 | 8 | 27 |
| Downregulated | | | 2 | 1 | 5 | 0 |
| Not significantly affected | | | 1 | 7 | 15 | 1 |

Changes in gene expression were statistically significant unless otherwise noted. The sequences reported met our post hoc filter criteria at one or more time points. Some of the genes in this table can be classified into more than one major functional category. NS, not significant. *Effect was statistically significant in the prior in vitro study but did not meet that study's post hoc filter criteria (listed in MATERIALS AND METHODS).

Table 7. *Control sequences*

| Common Name(s) | GenBank No. | Fold-Change (Relative to Control) | | |
|---|---|---|---|---|
| | | At presentation | After cooling | At follow-up |
| Cyclophilin A | X52851 | 1.1 | 0.94 | 1.2 |
| β-actin, 5′ sequence | X00351 | 0.98 | 1.1 | 1.6* |
| β-actin, middle sequence | X00351 | 1.2* | 1.2* | 1.7* |
| β-actin, 3′ sequence | X00351 | 1.2* | 1.3* | 1.5* |
| GAPDH, 5′ sequence | M33197 | 1.0 | 1.3 | 1.4 |
| GAPDH, middle sequence | M33197 | 1.0 | 1.5* | 1.4* |
| GAPDH, 3′ sequence | M33197 | 1.1 | 1.5* | 1.5* |
| Ribosomal protein L41 | Z12962 | 0.94 | 1.0 | 1.2 |
| Ribosomal protein S29 | AI541542 | 1.0 | 1.0 | 1.1 |
| Large ribosomal protein P1 | M17886 | 0.71† | 0.84 | 1.1 |
| Ribosomal protein S12 | AA977163 | 0.84† | 0.86 | 0.90 |
| Ribosomal protein L38 | Z26876 | 0.76 | 0.81 | 1.0 |

The list includes β-actin, GAPDH, cyclophilin A, and the 5 ribosomal protein mRNA sequences most highly expressed in the controls. *$P \leq 0.010$. †$0.010 < P < 0.0167$.

previous in vitro study in PBMCs was greatest for the samples obtained on the day of presentation (17–24%), as would be expected given the time point used in the in vitro study (2 h 40 min after heat shock). Importantly, the observed differences between the in vivo and in vitro responses suggest that, as might be anticipated from a multifactorial process, EHI also involves pathways other than those affected by in vitro heat shock in serum-free media. The molecular data reported here likely reflect not only the direct cellular effects of thermal stress on PBMCs but also the responses of PBMCs to the humoral or other signals detectable by cells traveling through tissues reacting to thermal stress.

It is noteworthy that many of the sequences most highly increased by EHI in this report are interferon-inducible genes (Table 8). Interferons-α and -γ have been variably reported to be elevated in exertional heat illness (reviewed in Ref. 2), and these immune modulators are known to be capable of producing flu-like syndromes, at least when administered at pharmacological doses (1, 15). As is commonly observed in EHI (5, 26), all of our cases reported feeling ill in the days leading up to the acute events, and at least one of the case subjects had

Table 8. *Non-HSP genes most strongly upregulated by EHI*

| Gene Type | Common Name(s) | GenBank No. | Fold-Change (Subjects/Controls) | | | Effect of In Vitro Heat Shock |
|---|---|---|---|---|---|---|
| | | | At presentation | After cooling | At follow-up | |
| | *Maximally upregulated at presentation* | | | | | |
| Immune function | GZMB; granzyme B; granzyme 2 | M17016 | 5.6 | 1.8 (NS) | 2.3 | Down |
| Phagocytosis | PSR; Phosphatidylserine receptor | AI950382 | 5.2 | 2.9 | 1.6 (NS) | Up |
| Membrane transport | SLC15A1; Solute carrier family 15 (oligopeptide transporter), member 1; peptide transporter HPEPT1; PEPT1 | AB001328 | 5.8 | 1.0 (NS) | 4.5 | NS |
| | *Maximally upregulated after cooling* | | | | | |
| Cell adhesion | Cadherin | L43366 | 7.0 | 7.2 | 6.3 | NS |
| | Ninjurin 1; nerve injury-induced protein-1; NINJ-1 | U91512 | 1.2 (NS) | 7.2 | 3.3 | NS |
| Histone | H2AFO; H2A histone family, member O | AI885852 | 11 | 22 | 18 | NS |
| Hormone | Adrenomedullin precursor | D14874 | 3.2 | 5.3 | 2.4 | Down |
| Immune function/ immunoglobulin receptor | FCGR1A; Fc fragment of IgG, high affinity Ia, receptor for (CD64); CD64; Fc-gamma receptor IA1 | M63835 | 3.4 | 11 | 8.6 | NS |
| Immune function/ complement receptor | C3aR1, complement component 3a receptor 1 | U62027 | 3.3 | 8.6 | 3.0 | Down |
| Immune function/ interferon inducible | IFI27; p27 | X67325 | 2.8 | 40 | 21 | NS |
| | G1P2; (clone IFI-15K)UCRP; IFI15; ISG15; interferon-stimulated protein, 15 kDa | M13755 | 5.0 | 6.7 | 5.1 | NS |
| | Mx1; myxovirus-resistance 1; interferon-inducible protein p78; MxA | M33882 | 4.3 | 5.6 | 5.3 | Down |
| | IFIT1; interferon-induced protein with tetratricopeptide repeats 1; interferon-induced protein 56 | M24594 | 4.0 | 5.6 | 5.3 | NS |
| | | M24594 | 3.3 | 5.0 | 4.1 | NS |
| Membrane function | Scramblase; phospholipid scramblase 1 | AB006746 | 4.5 | 5.3 | 4.3 | NS |
| Membrane transport | NEDD4L; neural precursor cell expressed, developmentally down-regulated 4-like | AB007899 | 2.8 | 5.0 | 1.3 (NS) | NS |
| Protein assembly | SCO2; SCO cytochrome oxidase deficient homolog 2 (yeast) | AL021683 | 4.5 | 5.1 | 5.1 | NS |
| RNA stability and degradation | OASL; 2′-5′-oligoadenylate synthetase-like; thyroid hormone receptor interactor 14; TRIP14; p59OASL | AJ225089 | 6.1 | 9.5 | 8.5 | NS |
| | | L40387 | 2.4 (NS) | 5.0 | 3.8 | NS |
| Unknown | AB000115; C1orf29; chromosome 1 open reading frame 29 | AB000115 | 6.2 | 7.8 | 5.7 | NS |
| | AIP1; atrophin-1 interacting protein 1 | AB014605 | 2.8 | 5.3 | 0.49 (NS) | NS |
| | *Maximally upregulated at follow-up* | | | | | |
| Cell growth, proliferation, and differentiation | PPP2R1B; protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform | M65254 | 2.3 | 8.2 | 12 | NS |
| Growth factor | ECGF1; endothelial cell growth factor 1 (platelet-derived) | M63193 | 2.7 | 3.5 | 5.4 | Down |
| Immune function/ interferon inducible | cig5; viperin | AF026941 | 9.9 | 20 | 20 | NS |
| | IFI 60; IFIT 4; interferon-induced protein with tetratricopeptide repeats 4 | AF026939 | 9.1 | 16 | 19 | Down |
| | IFIT2; interferon-induced protein with tetratricopeptide repeats; 2G10P2; IFI54; cig42; IFI-54; GARG-39; ISG-54K | M14660 | 6.8 | 8.5 | 9.2 | Down* |
| | CXCL10; chemokine (C-X-C motif) ligand 10; interferon-inducible cytokine IP-10 | X02530 | 1.4 (NS) | 1.2 (NS) | 8.4 | NS |
| | IFITM3; Interferon induced transmembrane protein 3 (1-8U) | X57352 | 4.2 | 5.5 | 6.9 | NS |
| Immune function/ complement cascade | Complement component C1 inhibitor | X54486 | 4.3 | 5.4 | 7.6 | NS |
| Metabolism | PC; pyruvate carboxylase | S72370 | 1.2 (NS) | 2.3 | 7.7 | Up* |
| Oxygen transport | HBE1; Hemoglobin, epsilon 1 | AI349593 | 3.2 | 3.9 | 5.1 | NS |
| Signal transduction | Tyrosine phosphatase DKFZP566K0524 | AL050040 | 5.7 | 3.4 | 7.3 | NS |
| | ADORA2B; adenosine A2b receptor | X68487 | 3.2 | 3.6 | 6.0 | NS |
| Translation termination/ interferon inducible | WARS; tryptophanyl tRNA synthetase | X59892 | 2.1 | 2.2 | 5.3 | NS |
| Unknown | ZAP3; ZAP3 protein | L40396 | 5.3 | 3.9 | 8.0 | NS |
| | SPUF; Secreted protein of unknown function | AA883101 | 4.4 | 4.1 | 5.4 | NS |

Included are all genes that met our post hoc filter criteria and that showed an increase in expression of 5-fold or greater over controls at any time point. NS, change in expression is not statistically significant. Effect of in vitro heat shock refers to the results of our laboratory's previous experiment with normal PBMCs (29). *Effect was statistically significant in the prior in vitro study but did not meet that study's post hoc filter criteria.

Table 9. *Non-HSP genes most strongly downregulated by EHI*

| Gene Type | Common Name(s) | GenBank No. | Fold-Change (Subjects/Controls) | | | Effect of In Vitro Heat Shock |
|---|---|---|---|---|---|---|
| | | | At presentation | After cooling | At follow-up | |
| | *Maximally downregulated at presentation* | | | | | |
| Cell growth, proliferation, and differentiation | MAL; mal, T-cell differentiation protein | X76220 | 0.13 | 0.29 | 0.22 | Up* |
| Immune function | FCER1A; Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide | X06948 | 0.039 | 0.13 | 0.21 | NS |
| Membrane transport | MS4A1; membrane-spanning 4-domains, subfamily A, member 1; CD20 antigen | X07203 | 0.090 | 0.37 | 0.52 (NS) | NS |
| Transcription | DRAP1; DR1-associated protein 1 (negative cofactor 2 alpha); Dr1-associated corepressor, mRNA sequence | U41843 | 0.13 | 0.39 | 0.15 | NS |
| | MYC; V-myc myelocytomatosis viral oncogene homolog (avian); c-myc | V00568 | 0.19 | 0.50 | 0.58 | NS |
| Unknown | TBC1D4; TBC1 domain family, member 4; KIAA0603 | AB011175 | 0.073 | 0.32 | 0.48 (NS) | NS |
| | *Maximally downregulated after cooling* | | | | | |
| Cell growth, proliferation, and differentiation | JAG1; jagged 1 (Alagille syndrome) | U77914 | 0.75 (NS) | 0.10 | 1.9 | NS |
| Immune function | SH2D1A; SH2 domain protein 1A, Duncan's disease (lymphoproliferative syndrome) | AL023657 | 0.71 (NS) | 0.053 | 0.54 (NS) | NS |
| Metabolism | AGL; amylo-1,6-glucosidase, 4-alpha-glucanotransferase (glycogen debranching enzyme, glycogen storage disease type III) | U84011 | 0.39 | 0.20 | 0.34 | NS |
| Neurotransmitter synthesis | GAD1; glutamate decarboxylase 1 (brain, 67 kDa) | M81883 | 0.40 (NS) | 0.088 | 0.44 (NS) | NS |
| Signal transduction | ARHH; Ras homolog gene family, member H | Z35227 | 0.54 (NS) | 0.19 | 0.48 | Down* |
| Transcription | ZNF85; zinc finger protein 85 (HPF4, HTF1) | U35376 | 0.89 (NS) | 0.077 | 0.61 (NS) | NS |
| Unknown | KIAA0982 | AB023199 | 0.61 | 0.16 | 0.69 | Up* |
| | *Maximally downregulated at follow-up* | | | | | |
| Apoptosis | APG5L; APG5 autophagy 5-like (*Saccharomyces cerevisiae*); ASP; apoptosis specific protein | Y11588 | 0.41 | 0.52 | 0.15 | Up* |
| Cell growth, proliferation, and differentiation | G0S2; Putative lymphocyte G0/G1 switch gene | M69199 | 0.033 | 1.4 (NS) | 0.018 | Down* |
| | COIL; Coilin; P80-coilin | U06632 | 0.69 (NS) | 0.26 (NS) | 0.11 | Down* |
| | ZRF1; zuotin related factor 1; MPP11; MPHOSPH11; M-phase phosphoprotein 11 | X98260 | 0.29 (NS) | 0.29 (NS) | 0.13 | NS |
| | PLAGL1: pleiomorphic adenoma gene-like 1 | U81992 | 1.0 (NS) | 0.62 | 0.19 | NS |
| Cytoskeleton | AB026190; Kelch motif-containing protein | AB026190 | 0.45 | 0.68 (NS) | 0.13 | NS |
| | TUBGCP3: tubulin, gamma complex-associated protein 3; GCP3; spindle pole body protein | AF042378 | 0.33 | 0.56 | 0.14 | Up |
| Immune function | PF4V1; platelet factor 4 variant 1; PF4-ALT | M26167 | 0.50 (NS) | 0.91 (NS) | 0.031 | NS |
| | TACTILE; T-cell-activated increased late expression | M88282 | 0.11 | 0.12 | 0.041 | Down |
| Membrane traffic and receptor sorting | STX8; Syntaxin 8 | AF036715 | 0.54 | 0.53 | 0.11 | NS |
| Membrane transport | KPNB3; karyopherin (importin) beta 3 | Y08890 | 0.34 | 0.49 | 0.066 | NS |
| Metabolism | DLAT; dihydrolipoamide *S*-acetyltransferase (E2 component of pyruvate dehydrogenase complex) | Y00978 | 0.63 (NS) | 0.60 (NS) | 0.12 | NS |
| | NNT; nicotinamide nucleotide transhydrogenase | U40490 | 0.47 | 0.33 | 0.18 | NS |
| Protein degradation | CTSS; cathepsin S | M90696 | 1.1 (NS) | 0.62 | 0.18 | NS |
| RNA processing | NUFIP1; nuclear fragile X mental retardation protein interacting protein 1 | AL049842 | 0.13 | 0.39 | 0.087 | Up* |
| Signal transduction | PRKCBP1; protein kinase C binding protein 1; RACK7; receptor for activated C-kinase 7 | W22296 | 0.83 (NS) | 0.41 (NS) | 0.13 | NS |
| | MAP3K12; mitogen-activated protein kinase kinase kinase 12 | U07358 | 0.33 | 0.42 | 0.14 | NS |
| | SMG1; PI-3-kinase related kinase SMG-1 | AI610467 | 0.66 (NS) | 0.38 (NS) | 0.20 | NS |
| Unknown | Hypothetical protein FLJ13110 | AL080222 | 0.59 (NS) | 0.49 (NS) | 0.032 | NS |
| | HZF12; zinc finger protein 12 | AL096747 | 0.10 | 0.21 (NS) | 0.096 | Down* |
| | KIAA 1718 | AC004849 | 0.32 | 0.41 (NS) | 0.14 | NS |
| | KIAA 0648 | AB014548 | 0.27 | 0.38 (NS) | 0.14 | NS |

Included are all genes that met our post hoc filter criteria and that showed a decrease in expression of at least 0.2-fold over controls at any time point. NS, change in expression is not statistically significant. Effect of in vitro heat shock refers to the results of our laboratory's previous experiment with normal PBMCs (29). *Effect was statistically significant in the prior in vitro study but did not meet that study's post hoc filter criteria.

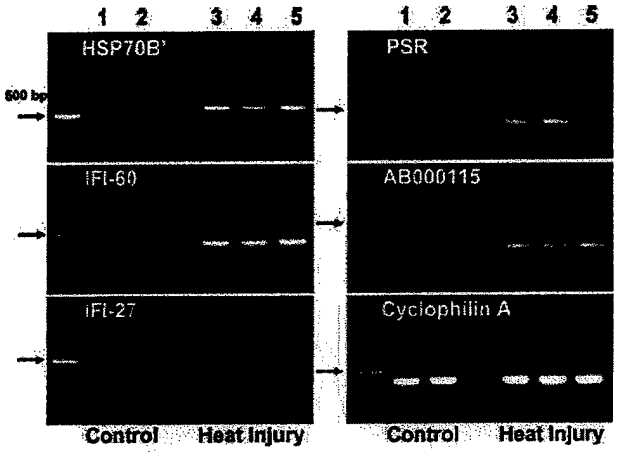


Fig. 2. Confirmatory RT-PCR. The controls are from *subject B*, taken before (*lane 1*) and after (*lane 2*) participation in a field-training exercise. The heat-injury samples are from *subject 2*, taken at presentation (*lane 3*), after cooling (*lane 4*), and at follow-up (*lane 5*). Sequences studied were heat shock protein (HSP) 70B'; interferon-induced protein 60 (IFI-60); interferon-α-inducible protein 27 (IFI-27); phosphatidylserine receptor (PSR); chromosome 1 open reading frame 29 (AB000115); and cyclophilin A. The ladder bands occur in 100-bp increments. The arrows point to the bright 500-bp band in each ladder.

firm evidence of an interferon-associated viral process (mononucleosis) on clinical evaluation. The finding of increased expression of interferon-induced genes at the time of presentation may simply be a molecular correlate of antecedent viral infections in our cases but may also identify candidate mediators that might account for the apparent association of EHI with prodromal symptoms suggestive of viral illness. Alternatively, at least some of the increases in expression of these sequences might reflect heat-induced increases in plasma levels of interferons. Further research will be required to establish whether the pathways associated with these sequences participate in the pathophysiology of EHI or whether their increased expression merely serves as markers that identify individuals at increased risk of EHI. Importantly, our microarray evidence to date suggests that the interferon-induced sequences (Table 8) are not directly upregulated by heat itself, because their expression was not significantly affected in our laboratory's previous in vitro heat shock experiment, and, indeed, at least three were found to be downregulated in vitro (29).

A comparison of the present work to our laboratory's previous in vitro work suggests that, as is generally believed, there is a cell type and stressor nonspecific component to the response to stress at the level of gene expression. This nonspecific component includes well-known stress genes such as ubiquitin C, HSP47/colligin 2, and dual-specificity phosphatase-1. The limited microarray evidence our laboratory has produced to date (including the present work) suggests that the number of genes involved in this nonspecific component may be a smaller fraction of the genomic response than previously thought, amounting to no more than ~10–15% of the total number of genes affected by any given environmental stress (27).

Our work confirms and extends, in important respects, the findings made in a mouse model of thermal stress reported recently by Zhang et al. (32). Among other findings, these authors reported that young mice exposed to two sequential thermal stresses had significant changes in liver gene expression in four principal categories: stress response genes (including HSPs); cell growth, death, and signaling-related genes; antioxidant enzymes and drug metabolism enzymes; and DNA/RNA/protein repair-related genes. Although we found genes related to immune function to be the largest single category affected by EHI (as might be expected from the fact that we studied PBMCs rather than other cell types), we also found a large number of genes to be affected by EHI that are functionally thought to be involved in the cell stress response, signal transduction, metabolism/redox control, and cell growth, proliferation, differentiation, and apoptosis (Tables 8 and 9). This suggests that a number of the functional responses to thermal stress at the level of RNA expression are conserved across species and cell types and adds support to the use of rodent models for the study of heat-related illnesses.

There are several important limitations to this study. First, as an observational rather than an experimental study, the general applicability of its conclusions are limited by the low number of subjects and the presence of significant intersubject variation in severity of illness, prodromal symptoms, clinical course, and timing of sample collection. Accordingly, we are unable to determine the extent to which particular individual sequences might influence severity of disease from the present data. Nonetheless, although the degree of severity was broad, all subjects met clinical criteria for EHI (11). Second, although our controls were matched for gender, ethnic origin, and both season and year of exposure, they were not drawn in parallel with the cases during the events that precipitated EHI. It is, therefore, possible that some of the gene-expression changes identified in our subjects suffering from EHI reflect the effects of variables, such as different types or levels of physical activity, and are not pathophysiologically related to EHI itself. Indeed, increases in leukocyte HSP expression have previously been described in individuals undergoing intense physical activity (6–8, 24), although it can be difficult in these studies to separate the effects of exercise from the effects of exercise-induced increases in body temperature. Third, the low number of replicates (only 2 control chips) coupled with the high number of sequences tested (~12,600) makes it likely that some of the changes in expression we report as significant represent false-positive results. We have attempted to reduce this by application of strict post hoc filter criteria. Fourth, it is possible that some of the changes in gene expression noted reflect a shift in the relative distributions of different subpopulations of circulating PBMCs rather than changes in expression within the same cells. Although this is unlikely to be the case for molecules such as HSPs (which are almost universally expressed by cells in response to thermal stress), it could account for some of the apparent changes in expression of genes known to be highly expressed by specific cell types [e.g., granzyme B, which is selectively expressed by cytolytic T cells and natural killer cells (30)]. Finally, we do not have measurements of plasma interferon levels.

In summary, this is the first large-scale survey of gene expression changes related to EHI or EHS. Our findings suggest that EHI produces gene expression changes that are far more extensive than previously realized. The gene expression signatures that occur during EHI include a classic heat shock response, a small nonspecific cell stress response, and a re-

sponse that cannot be fully explained by our previous in vitro work. This last component involves a significant number of interferon-inducible sequences and might, therefore, account for the known association of EHI with prodromal flulike symptoms. Finally, PBMCs provide an easily obtainable tissue to study gene expression with EHI and EHS.

## ACKNOWLEDGMENTS

The authors thank Michael Cullivan for assistance in sample preparation and Dr. Richard Pratt, whose laboratory performed the gene chip hybridizations and first-pass MAS software analysis. The authors also thank Dr. Jacques Reifman, Dr. Sorin Draghichi, and the US Army Medical Research and Material Command Bioinformatics Cell for valuable discussions pertaining to the statistical approach used in this manuscript.

The views, opinions, and findings contained in this publication are those of the authors and should not be construed as an official US Department of the Army or Department of the Navy position, policy, or decision, unless so designated by other documentation.

For protection of test subjects, the investigators adhered to the protections of 45 CFR 46.

Approved for public release; distribution unlimited.

## GRANTS

This study was sponsored in part by the Navy Bureau of Medicine and Surgery Clinical Investigation Program No. P00-L-H00000-025:A and by National Heart, Lung, and Blood Institute Grant HL-64104.

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